Elastase and Exacerbation of Neutrophil Innate Immunity are Involved in Multi-Visceral Manifestations of COVID-19

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Abstract

Background: Many arguments suggest that neutrophils could play a prominent role in COVID-19. However, the role of key components of neutrophil innate immunity in severe forms of COVID-19 has deserved insufficient attention. We aimed to evaluate the involvement of Neutrophil Elastase, histone-DNA, and DNases in systemic and multi-organ manifestations of COVID-19. Methods: We performed a multicenter study of markers of neutrophil innate immunity in 155 cases consecutively recruited in a screening center (ambulatory subjects), local hospitals, and two regional university hospitals. The case were evaluated according to clinical and biological markers of severity and multi-organ manifestations and compared to 35 healthy controls. Results: Blood Neutrophil Elastase, histone-DNA, myeloperoxidase-DNA and free dsDNA were dramatically increased, and DNase activity decreased by 10-fold, compared to controls. Neutrophil Elastase and histone-DNA were associated with intensive care admission, body temperature, lung damage and markers of cardiovascular outcomes, renal failure and increased IL-6, IL-8 and CXCR2. Neutrophil Elastase was an independent predictor of the computed tomography score of COVID-19 lung damage and the number of affected organs, in multivariate analyses. The increased blood concentrations of NE and neutrophil extracellular traps were related to exacerbation of neutrophil stimulation through IL8 and CXCR2 increased concentrations and increased serum DAMPs, and to impaired degradation of NETs as a consequence of the dramatic decrease of blood DNase activity. Conclusion: Our results point out the key role of neutrophil innate immunity exacerbation in COVID-19. Neutrophil Elastase and DNase could be potential biomarkers and therapeutic targets of severe systemic manifestations of COVID-19.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) produces thromboembolic events and multi-organ manifestations, including in heart, liver and kidneys[1, 2]. They result in part from the exacerbation of innate immunity [3] and a cytokine storm produced by lymphocyte and macrophage activation [4-6]. Arguments suggest that neutrophils also play a prominent role in the severe and life-threatening forms of the disease [6-9]. However, the involvement of neutrophils in systemic and multi-organ outcomes of COVID-19 has deserved insufficient attention[8].

Neutrophils have an arsenal of defensive strategies that include the release of antimicrobial granules and neutrophil elastase (NE), and the formation of neutrophil extracellular traps (NETs) [6, 8]. NETs are histone-DNA components of dying neutrophils involved in the host defense against pathogens [6, 8]. A study reported that two markers of NETs, cell-free DNA and myeloperoxidase (MPO)-DNA, were increased in hospitalized COVID-19 patients compared to 30 controls and were correlated with C-reactive protein, D-dimer, lactate

dehydrogenase and absolute neutrophil count [9]. Another study from our group showed that MPO-DNA level is increased in the early phase of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, in ambulatory cases [10]. The homeostasis of circulating NETs is dependent on NE and DNase 1 and DNase 1L3[6]. Excessive NE and NETs in blood produces vascular and tissue lesions in acute viral pneumonia, which resemble those observed in COVID-19 infection [6, 11, 12]. Despite these evidences, whether NE, DNases and histone-DNA are involved in the multi-visceral manifestations of COVID-19 has not been evaluated to date.

We aimed to study the blood level of NE, total DNase activity, histone-DNA and other NET components in patients with COVID-19 infection according to disease severity and multi-organ manifestations. We found that NE was an independent predictor of the multi-organ injury produced by COVID-19. The release of NE and NETs was related to neutrophil activation by serum damage-associated molecular patterns (DAMPs) and IL-8/CXCR2 pathway.

MATERIAL AND METHODS

Recruitment of patients and clinical characteristics of the study population. The NICO study (Neutrophil innate Immunity in COvid-19) was registered by the Institutional Review Board of Clinical Research (DRCI) of the University Hospital of Nancy (N° 2020PI087) and approved by the Ethical committee. We performed a cross-sectional consecutive study of 155 positive patients recruited after informed consent in a screening center, local hospitals and departments of medicine and intensive care units of two university hospital centers in the highest pandemic period of COVID-19 in France. We followed the EQUATOR and BRISQ guidelines for the reporting clinical and biological data and the use of biological specimens. The infection was characterized by RT-PCR on nasal swab specimens. The 34 consecutive ambulatory subjects attended the screening center in the first week of the disease, as previously described. [10] We recruited the 121 patients hospitalized for COVID-19 in medical departments of local hospitals of the region of Tours, Regional University Hospitals of Marseille and Nancy. Among them, 13 were treated in intensive care units. We reported the clinical characteristics and outcomes of hospitalized patients listed in **Supplementary Table 1** . The scoring of lung damage was evaluated by computed tomography in 5 grades (CT score), as described [13].

Blood sampling and biological assessment. The sera were used after completion of biochemical testing ordered by the clinician. The remaining samples were stored in the same conditions among groups, at 20°C in the 24 hours following blood withdrawal. Routine biochemical markers were assayed in Cobas 8000 analyzers (Roche Diagnostics, Switzerland) and Atellica (Eschborn, Germany).

Measurement of elastase, MPO-DNA, DNA-histone complexes, dsDNA and cytokines in serum. Circulating levels of histone-DNA complexes were measured in serum by the Cell Death Detection ELISA kit (Roche Diagnostics, Sigma Aldrich, USA), as described previously[14]. Values were reported in 450 nm absorbance units. DNA-MPO complexes were measured by replacing the anti-histone antibody with a rabbit polyclonal antibody against myeloperoxidase (Merck, Darmstadt, Germany). NE concentration was determined using the Neutrophil Elastase Human ELISA Kit (Invitrogen). Values were reported in ng/mL. Cell-free dsDNA was quantified using the QuantiT PicoGreen® dsDNA Assay Kit, after subtraction of background (Invitrogen). IL-6, IL-8 and CXCR2 were assayed by ELISA kits from Reagentbio Ireland LTD, Dublin, Ireland, and TNFα by ELISA kit from R&D Systems Inc.USA, Minneapolis, Minnesota, USA.

Total Deoxyribonuclease (DNase) activity in serum by single radial enzyme diffusion (SRED) assay. DNase activity was quantified on agarose gel containing 0.13 $mg.mL^{-1}$ DNA from salmon sperm in a buffer containing 100 mM MES pH 6.5, 20 mM $MgCl_2$, 2 mM $CaCl_2$ and SYBR Safe DNA gel stain (Invitrogen, Life Technology) as described.[52,53]Two microliters of serum were located into wells. Gels were incubated for 24 hours at 37 °C in a humid chamber. The DNA-SYBR fluorescence was recorded with a fluorescence scanner.

Quantification of cell-bound NETs in neutrophils incubated with patient serum. To determine whether serum of COVID-19 patients influence cell-bound NETs, blood from one healthy donor was mixed (1:1 vol/vol) with patient sera prior to PMA treatment and incubated for 6 hours. Red cell lysis was achieved

by the addition of 1 mL of FACS lyse reagent (BD Biosciences; 1:10 dilution) for 5 min. Bovine serum albumin (BSA, 1 mL at 2%) in phosphate-buffered saline (PBS) was then added and the mixture was centrifuged at 1000 *g* for 10 min. The pellet resuspended in PBS was analyzed using a Gallios flow cytometer (Beckman Coulter, Villepinte, France). The gating strategy sequentially focused on (i) CD66 positive cells and then (ii) H3Cit-positive and MPO-positive cells.

Assay for in vitro NET DNA release of Neutrophils by patient's serum. Isolated neutrophils were resuspended in RPMI 1640 and 1×10^5 cells were seeded in 96-well tissue culture plates. Plates were incubated at 37°C to allow cells to adhere. Following stimulation with 100 µL of 0.1 ng/mL PMA for 4h, wells were incubated with 1 U/mL MNase or DNase1 (both from Worthington) for 10 min or 10 µL patient or control serum for 6 h, and then 2 mM EDTA was added to stop nuclease activity. The DNA content released in the supernatant was quantified using the QuantiT PicoGreen® dsDNA Assay Kit (Invitrogen). The amount of released DNA was considered as 100% in unstimulated neutrophils of healthy donors.

Statistical analyses. All quantitative variables are shown as the median and interquartile range (IQR, 25–75th percentile) and qualitative variables as percentages and 95% confidence interval (95% CI). Non-parametric tests were used when data distribution was not normal. Univariate analyses were performed using the chi-squared test or Fisher's exact test for categorical variables and the Mann-Whitney U and Kruskal-Wallis tests and Spearman's rank correlation for continuous variables. Post-hoc pairwise comparisons between subgroups were performed using the Conover test. We subsequently performed receiver operating characteristic (ROC) analysis to look for the optimal thresholds associated with disease outcomes [15]. Clinical and biological criteria were used to define organ injuries (see the Methods section in this article's Online Repository at*www.jacionline.org*). Bias-corrected and accelerated (BCa)-bootstrap interval after 10,000 iterations for the Youden index and its associated values were performed[16]. In step #2, using multivariable logistic regression analysis, we assessed whether the ROC-defined threshold defined for elastase (>593 ng/L) was associated with a 'number of affected organs [?]2' after accounting for potential confounders. We assessed model discrimination using ROC analysis (AUROC and 95% CI) and model calibration using the Hosmer and Lemeshow goodness-of-fit test and Nagelkerke R2 statistics [17]. In step #3, we assessed the association between elastase level (> or [?]593 ng/L) and patients' demographics, medical histories, and disease outcomes. Statistical analyses were performed using MedCalc, version 19.1 (MedCalc Software, Ostend, Belgium) and Stata SE, version 12.1 (College Station, Texas, US), based on a two-sided type I error with an alpha level of 0.05.

RESULTS

NE, MPO-DNA, histone-DNA are dramatically increased and DNase activity was dramatically decreased in COVID-19 ambulatory and hospitalized patients. The blood level of NE, MPO-DNA, histone-DNA and dsDNA of ambulatory and hospitalized COVID-19 patients were dramatically higher than those reported in control subjects. In contrast, the global DNase activity was 10-fold lower in COVID-19 cases. (**Figure 1**). The serum concentration of NE and NET components were not associated with age and sex among groups. The 34 ambulatory cases had a mean age (+/-SD) of 42+/-17 years and a sex ratio (M/F) at 0.88. All had at least two symptoms among fever, dry cough, and dyspnea for less than one week. None had a severe form at this step of the disease. The clinical characteristics and biological findings of hospitalized COVID-19 patients are reported in the **Supplementary Tables S1**and **S2** and those of the 35 healthy controls in**Supplementary Table S3** . The concentration of NE was significantly higher in hospitalized *vs.* ambulatory cases. We observed a higher concentration of NE in patients treated in intensive care *vs* . local hospitals and medical departments of university hospitals (**Figure 1**). We also observed normal serum concentrations of α1 antitrypsin (AAT), the main blood inhibitor of NE, in hospitalized cases (**Supplementary Table S2**). NE concentrations were distributed in two clusters in ambulatory cases and patients hospitalized in medical department of regional university hospitals. The clusters with the highest concentrations had similar values than those reported in intensive care units (**Figure 1**). Similarly, the concentration of dsDNA was higher in patients admitted in intensive care units (**Figure 1**). We observed highly significant associations of NE with histone-DNA and MPO-DNA (**Figure 2**), while the concentration

of dsDNA was associated only with histone-DNA (P=0.03). We also found significant associations of NE with interleukin-6 (IL-6), IL-8 and CXCR2, but not with TNFα (**Figure 3**).

NE, histone-DNA and total DNase activity are associated with severity and multi-visceral manifestations of COVID-19. We reported significant associations of NE with SaO2 at hospital admission, leukocytes, neutrophils, neutrophil to lymphocyte ratio, LDH, markers of cardiovascular and thrombotic risk, including Troponin-T (cTnT), fibrinogen and D-dimer, and markers of renal failure, including urea and creatinine (**Table 1** and **Figure 3)** . Significant association of histone-DNA was reported with the computed tomography score (CT score) of lung damage [13], markers of cardiovascular and thrombotic risk,[13] including cTnT and fibrinogen, markers of renal failure, including urea and creatinine and markers of inflammation, including C-reactive protein, ferritin and body temperature (**Table 1** and **Supplementary Figure S1**). In contrast, MPO-DNA was associated only with leukocytes (P=0.005) and neutrophils (P=0.004), and at weaker significance, with D-Dimer (P=0.045) and fibrinogen (P=0.047). Total DNase activity was associated with alkaline phosphatase (PAL) and total bilirubin while dsDNA was associated only with ferritin (**Table 1**).

Diagnostic accuracy of NE, MPO-DNA, histone-DNA, dsDNA, DNase activity and related cytokines for detecting disease outcomes in receiver operating characteristic (ROC) analyses . We performed ROC analyses to assess the diagnostic accuracy of NE, MPO-DNA, and histone-DNA for the prediction of disease-related outcomes (**Supplementary Table S4 and Figure 4**). NE had significant thresholds for admission into intensive care units, occurrence of respiratory failure, blood oxygen saturation <85%, and the presence of at least two affected organs (**Figure 4**). The latter was best predicted with a cut-off at 593 ng/mL of NE. Histone-DNA had significant thresholds for admission into intensive care units, kidney injury, and blood oxygen saturation <85%. MPO-DNA had significant thresholds for heart decompensation, kidney injury, respiratory failure, and blood oxygen saturation <85%. The ROC analyses identified also very significant cut-offs for IL-6, IL-8, and CXCR2 for admission into intensive care units, heart decompensation, kidney injury, and respiratory failure. The same observation was made for CXCR2.

Associations between NE >593 ng/mL and patients' characteristics and outcomes. In univariate analyses, NE >593 ng/mL was significantly associated with CT score, blood oxygen saturation, respiratory failure, presence of at least two affected organs and admission into the intensive care unit (**Supplementary Table S5**). We retained chronic kidney disease, diabetes, and hypertension as potential confounders in multivariable analyses (**Supplementary Table S6**). In the multivariable models that were used to account for the collinearity issue, NE >593 ng/mL was independently associated with CT score and presence of at least two affected organs (**Supplementary Table S7**). The optimal multivariable model had an area under the receiver operating characteristics (AUROC) of 0.876 (95% CI, 0.758–0.950) and a percentage of cases correctly classified of 85%. We performed concordance analyses between cut-offs of NE and cytokines in the prediction of least two affected organs. We reported a 70.4% and 72.7% concordance of NE with CXCR2 and IL-6, respectively (**Figure 2**).

Patient sera decrease the retention of NETs and increase the release of dsDNA in isolated control neutrophils . We studied cell-bound NETs of control neutrophils incubated with patient sera using flow cytometry. We observed a decreased retention of cell-bound NETs, which reflected an increased release, from cells incubated with patient*vs.* control sera. We further studied the release of dsDNA from control neutrophils produced by patient sera. We observed an increased release of dsDNA from neutrophils incubated with patient *vs.*control sera (**Figure 5**). The inhibition of NE and other serum serine proteases with 10 μM aprotinin had a weak inhibition effect on dsDNA release. Taken together, these data suggested that the sera from patients increased the release of components of NETs, with a limited role of NE.

DISCUSSION

Our study showed that NE, global DNase activity and components of NETs are involved in the early and later steps of COVID-19. NE and histone-DNA were associated with clinical manifestations and biomarkers related to pulmonary damage, cardiovascular manifestations, renal insufficiency, and inflammation. Elastase

>593 ng/mL was an independent predictor of multi-organ injury in multivariate analysis.

The dramatic increase of blood levels of NE and NETs in ambulatory cases show their involvement in the early host response to SARS-CoV-2, as previously observed for other viral pneumonia [4, 6, 11, 18]. Alveolar epithelial cells from lungs infected with influenza virus stimulate NETosis [19]. However, there is a debate on the detrimental *vs.* protective effects of NETs in acute respiratory distress syndrome (ARDS) [20, 21]. The citrullinated H3 and cell-free DNA reflect the production of extracellular traps by neutrophils, granulocytes and monocytes[21, 22]. The dramatic increase of blood concentration of NE reflects more specifically the activation of neutrophils[22, 23]. Of note, NE concentration correlates better with alveolar inflammation than neutrophil count, in acute respiratory distress syndrome [23, 24].

NE, but not histone-DNA and MPO-DNA, was an independent predictor of multi-organ damage in COVID-19 patients. This result reflects the prominent role of NE in mechanisms of neutrophil innate immunity[22]. NE is dispensed in tissues and blood by degranulation or release with NETs [22, 25-27]. The induction of ROS by viral infection activates MPO, which then activates the release of NE [27, 28]. The highly significant correlation between NE and histone-DNA and MPO-DNA illustrates the role of NE in NET formation. Upon neutrophil stimulation, NE translocates to the nucleus, where it participates in histone degradation before it releases with the DNA/chromatin material of NETs [6, 28]. Importantly, NE associated with NETs remains active and escapes the endogenous anti-protease activity of AAT [29]. NE can produce tissue damages in lung, heart, liver, vessels and kidney and exerts prothrombotic effects in viral infection [6, 24, 29-35]. The degradation of extracellular matrix (ECM) components by NE produces the same lung and vascular injuries as those observed in autopsy specimens of COVID-19 patients [32-37]. The associations of NE and histone-DNA with SaO_2 at hospital admission and CT score of lung damage of COVID-18 are consistent with their effects in other lung viral infections [6, 12, 38]. Their associations with troponin-T, D-dimer and fibrinogen suggest their role in the prothrombotic effects reported in COVID-19[37-39]. The myocardial injury during COVID-19 is not clearly understood [40-42]. The mechanisms include direct viral infection, thrombosis, microvascular and myocardial injury related to reduced oxygen delivery and release of cytokines[32, 41, 43]. The increased cTnT associated with NE could be secondary to coronary thrombosis and myocardial infarction and/or myocarditis [32, 41, 42]. NE and NETs could also contribute to the association of heart injury through systemic effects in kidney and other organs [2, 43, 44]. The associations of NE and histone-DNA with urea and creatinine suggest their involvement in the acute kidney injury reported in about 30% of COVID-19 patients[31, 45-47].

Our study contributes to a better understanding of pathological mechanisms of COVID-19 by pointing out the key role of the disruption of neutrophil innate immunity during and after viral replication, as summarized in **graphical abstract** . IL-6, IL-8 and TNFα account among the cytokines predominantly associated with COVID-19 severity[48]. IL-6 and IL-8 are produced upon NF-ϰB activation of infected alveolar macrophages through mechanisms that probably involve Bruton Tyrosine Kinase (BTK) [49]. This is illustrated by the promising clinical effects produced by the BTK inhibitor acalabrutinib in patients with severe COVID-19[49]. IL-8 act as neutrophil-activating chemokine through its binding to CXCR2, which is a major chemokine receptor of neutrophils [50]. Therefore, the dramatic increased of NE in severe COVID-19 may be related to neutrophil activation by the IL8/CXCR2 pathways [51]. LDH, ferritin and D-dimer are highly correlated with NE and could reflect the macrophage activation of COVID-19 [47, 52]. Conversely, NE and NETs could also play a role in macrophage activation and the processing of proinflammatory cytokines [3, 6, 34-36]. We reported a dramatic decrease of global DNase activity in serum which could participate in NETosis through decreased degradation of circulating chromatin-DNA fragments (**graphical abstract**)[53, 54-56]. It is noteworthy that a*Dnase1−/− Dnase1l3−/−* mouse model exposed to pathogens produces lung lesions similar to those observed in patients with respiratory distress and/or sepsis and autopsies of COVID-19 patients[38, 57]. Consistently, a recent study reported a decreased activity of DNase I in bacterial and viral pneumonia in children [58].

The sera of COVID-19 patients decreased the cell retention of NETs and increased the release of dsDNA of neutrophils from healthy donors. Similar results have been previously obtained with COVID19 serum using the cell dye SYTOX Green to quantify NETs [9]. These results show that the NETosis can be triggered by endogenous stimuli released in blood by injured tissues such as DAMPs, including free dsDNA[59].

Our study opens up therapeutic perspectives. The excessive NE activity reported in our study suggests evaluating the use of new generation potent NE inhibitors, including lonodelestat (POL6014), alvelestat, CHF6333, and elafin in COVID-19 [8,25]. The dramatic decrease of DNase reported in our study also suggests evaluating the use of recombinant deoxyribonuclease I (dornase alfa) and/or DNase 1L3 [60-62]. One expected effect is the release of NE from degraded NETs, with increased free NE in blood and subsequently improved efficacy of NE inhibitors[28]. For this reason, we think that the association of DNase inhibitors with NE inhibitors should be considered in the treatment of COVID-19. Another therapeutic option to be considered could be the use of inhibitors of CXCR2 to block the neutrophil recruitment and activation.

Our study suffered limitations. We could not systematically report all biomarkers in all patients. The recruitment and cross-sectional evaluation did not allow us to study the kinetics of NE and NETs by the follow-up of patients. However, we were able to compare the concentration of NE and NETs in ambulatory symptomatic patients recruited during the first week of COVID-19 and patients hospitalized during the second to fourth week of the disease. The multicentric recruitment in local hospitals and in regional university hospitals was a strength of our study for evaluating cases with contrasted disease severity. The exhaustive recording of clinical data allowed us to perform multivariate analysis of the association of NE and NETs with severity and multi-organ damage of COVID-19, after forced adjustment for medical history.

In conclusion, our study points out the involvement of NE, DNase activity, and NETs as components of the innate immune defense of neutrophils against virus infection during the first phase of COVID-19 and their key role in the severity of the acute respiratory distress syndrome and cardiovascular, renal and inflammatory systemic manifestations in the later step of the disease. They suggest evaluating NE, DNases 1 and NETs as potential therapeutic targets.

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FIGURE LEGENDS

Figure 1. Neutrophil elastase (NE), myeloperoxidase – DNA (MPO-DNA), histone-DNA and (*b*) cell-free dsDNA and total DNase activity in serum of COVID-19 positive cases. Symptomatic ambulatory cases were recruited in screening centers and hospitalized patients in local hospitals and in medical departments and intensive care units of two regional university hospitals. All values were expressed in interquartile range (IQR, 25–75th percentile) NE values: healthy controls 29.5 (23-55), ambulatory 422 (264-659), proximity hospital 405 (224-953), medical department regional hospital 775 (409-1424) and intensive care regional hospital 1366 (1122-1505), the P values between groups were: healthy vs ambulatory <0.0001; healthy vs proximity hospital <0.0001; healthy vs medical department <0.0001; healthy vs intensive care <0.0001; ambulatory vs proximity hospital 0.7870; ambulatory vs medical department 0.0028**.** ambulatory vs intensive care <0.0001; proximity hospital vs medical department 0.0003; proximity hospital vs intensive care <0.0001 and medical department vs intensive care 0.0239. MPO-DNA values: healthy controls 0.36 (0.23-0.45), ambulatory 1.30 (1.14-1.45); proximity hospital 1.38 (1.28-1.46), medical department regional hospital 1.32 (1.17-1.46), intensive care regional hospital 1.37 (1.32-1.48) and intensive care 0.13 (0.10-0.18), the P values between groups were healthy vs ambulatory <0.0001; healthy vs proximity hospital <0.0001; healthy vs medical department <0.0001; healthy vs intensive care <0.0001; ambulatory vs proximity

hospital 0.1237; ambulatory vs medical department 0.8589**.** ambulatory vs intensive care 0.1276; proximity hospital vs medical department 0.1463; proximity hospital vs intensive care 0.5811 and medical department vs intensive care 0.1482. Histone-DNA values: healthy controls 0.13 (0.10-0.17), ambulatory 0.91 (0.77-1.10), proximity hospital 1.19 (0.91-1.39), medical department regional hospital 1.15 (0.76-1.32) and intensive care regional hospital 1.40 (1.35-1.53), the P values between groups were healthy vs ambulatory <0.0001; healthy vs proximity hospital <0.0001; healthy vs medical department <0.0001; healthy vs intensive care <0.0001; ambulatory vs proximity hospital 0.0013; ambulatory vs medical department 0.0525**.** ambulatory vs intensive care <0.0001; proximity hospital vs medical department 0.2740; proximity hospital vs intensive care 0.0.0033 and medical department vs intensive care 0.0003. Cell-free dsDNA values: healthy controls 103 (76-138), proximity hospital 120 (90-178), medical department regional hospital 122 (94-207) and intensive care regional hospital 200 (124-286). DNAse activity values: healthy controls 29.25 (22.68-31.48), medical department regional hospital 2.24 (1.66-2.71) and intensive care regional hospital (2.06 (1.61-2.93). The thresholds (dotted lines) were evaluated in healthy controls recruited several months before the epidemy. The threshold of NE was estimated at 73 ng/mL and those of MPO-DNA at 0.562 AU (450 nm absorbance units), histone-DNA at 0.591 AU, total activity of DNase at 9.94 U/mL and serum dsDNA at 95.60 ng/mL*c***.** NE, MPO-DNA and histone-DNA according to the number of organs affected by COVID-19. Data from a , b and c were compared by the Mann-Whitney test.

Figure 2. Associations of neutrophil elastase (NE) with components of neutrophil extracellular traps (NETs), clinical features and biomarkers of severity and multi-visceral harm of COVID . Significant associations of NE were reported with blood concentration of myeloperoxidase (MPO)-DNA and histone-DNA, blood counts of neutrophils, neutrophil/lymphocyte ratio and blood biomarkers of multi-visceral harm, including urea, SaO2, troponin-T, lactate dehydrogenase (LDH), D-dimer, fibrinogen. Correlations were assessed by Spearman rank correlation.

Figure 3. Associations of neutrophil elastase (NE) with cytokines involved in COVID-19 pathological mechanisms . Significant associations of NE were reported with interleukin-6 (IL-6), IL-8 and neutrophil chemokine receptor CXCR2, but not with TNFα. Dotted lines represent the cut-offs reported for multi-organ damage in receiver operating characteristic (ROC) analyses. These cut-offs were used for the concordance analyses. **Correlations** were assessed by Spearman rank correlation.

Figure 4. Forest plot reporting the summary of the receiver operating characteristic (ROC) analyses to assess the diagnostic accuracy of neutrophil elastase (NE), myeloperoxidase (MPO)-conjugated DNA, histone-DNA, cell-free dsDNA and DNase activity for the prediction of disease-related outcomes. For each biomarker, a ROC analysis was performed using the following classification variables: intensive care unit admission, heart decompensation, liver injury, kidney injury, respiratory failure, blood oxygen saturation <85%, number of affected organs [?]2, and NEWS 2 score [?]5. For each ROC analysis, the summary results were reported using the area under the ROC curve, the 95% confidence interval, and the associated P -value (Supplementary Table 3).

Figure 5. Neutrophil innate immunity is a target of the systemic effects of COVID-19. *a*, *b*. NET formation and release of Neutrophil dsDNA by sera of COVID-19 patients. a . Representative data of flow cytometry experiments showing NET detection as citrullinated H3 (H3cit) and myeloperoxidase (MPO) double-positive neutrophils (top) and NE-positive neutrophils (bottom), after incubation of neutrophils with sera. b . Quantitative analysis of NET retention on neutrophils from a healthy donor incubated with control or patient sera. c . Quantification of DNA release from neutrophils of a healthy donor stimulated with PMA to induce NET formation and subsequently incubated with 10% serum from controls or patients in the absence or in the presence of aprotinin. The amount of released DNA was considered as 100% in unstimulated neutrophils from the donor.

TABLE

Table 1. Significant correlations between elastase, myeloperoxidase-conjugated DNA, and histone-DNA complex and clinical and biological patients' characteristics

Variables	n	Rho
Elastase	**Elastase**	**Elastase**
hs-c Troponin l (ng/L)	15	0.82 (0.53–0.94)
Fibrinogen (g/L)	31	0.56 (0.25–0.77)
Histone-DNA complex	121	0.55 (0.4–0.66)
Neutrophils (G/L)	107	0.50 (0.34–0.63)
Leucocytes (G/L)	108	0.49 (0.33–0.62)
D-dimer (µg/mL)	28	0.49 (0.13–0.73)
Myeloperoxidase-conjugated DNA	121	0.45 (0.29–0.58)
LDH (U/L)	53	0.44 (0.18–0.64)
Urea nitrogen (g/L)	87	0.34 (0.14–0.52)
C-reactive protein (mg/L)	97	0.21 (0.01–0.4)
Creatinine (mg/L)	110	0.21 (0.02–0.39)
Platelets (G/L)	107	0.21 (0.01–0.39)
Blood oxygen saturation (%)	73	-0.24 (-0.45—0.01)
Neutrophils on lymphocytes ratio	107	-0.33 (-0.5—0.15)
Myeloperoxidase-conjugated DNA	**Myeloperoxidase-conjugated DNA**	**Myeloperoxidase-conjugated DNA**
hs-c Troponin l (ng/L)	15	0.68 (0.23–0.89)
Elastase (ng/mL)	121	0.45 (0.29–0.58)
Histone-DNA complex	121	0.42 (0.26–0.56)
D-dimer (µg/mL)	28	0.38 (0–0.67)
Fibrinogen (g/L)	31	0.36 (-0.01–0.64)
Ferritin (µg/L)	36	0.36 (0.03–0.62)
LDH (U/L)	53	0.36 ()0.10–0.58
C-reactive protein (mg/L)	97	0.33 (0.13–0.50)
Leucocytes (G/L)	108	0.27 (0.08–0.44)
Neutrophils (G/L)	107	0.27 (0.08–0.45)
γ glutamyltransferase (U/L)	65	0.25 (0–0.48)
Total bilirubin (mg/L)	71	0.23 (-0.01–0.45)
Neutrophils on lymphocytes ratio	107	-0.20 (-0.38–0)
Histone-DNA complex	**Histone-DNA complex**	**Histone-DNA complex**
hs-c Troponin l (ng/L)	15	0.78 (0.44–0.93)
Fibrinogen (g/L)	31	0.64 (0.36–0.82)
Elastase	121	0.55 (0.4–0.66)
Ferritin (µg/L)	36	0.49 (0.19–0.71)
Myeloperoxidase-conjugated DNA	121	0.42 (0.26–0.56)
LDH (U/L)	53	0.38 (0.11–0.59)
CT score*	54	0.30 (0.02–0.53)
Urea nitrogen (g/L)	87	0.29 (0.08–0.48)
Creatinine (mg/L)	110	0.26 (0.08–0.44)
Temperature (°C)	70	0.25 (0.01–0.46)
C-reactive protein (mg/L)	97	0.21 (0.01–0.4)
ASAT (U/L)	92	0.21 (0–0.4)
Transferrin (g/L)	10	-0.83

* CT score: computed tomography score based on the visual quantitative evaluation of acute lung inflammatory lesions involving each lobe, which was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%), respectively.

Figure 1

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Figure 2

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Figure 3

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Figure 4

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Figure 5

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